Identification of glycosylations affected by androgen-regulated process on the surface of extracellular vesicles derived from PCa cell lines

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ABSTRACT

Introduction: Prostate cancer (PCa) is one of the leading death-causing cancers in males. Lack of sensitivity of the current diagnostic approach created an opportunity for a new diagnostic method. Altered glycosylation is a well-known phenomenon in cancer and can be used as an essential hint for identification of biomarkers of cancer. In this research, the aim is to identify glycosylation from PCa cell line on extracellular vesicles (EVs) which is regulated by the androgen hormone using a variety of lectins.

Methods: The LNCaP, a PCa cell line, was treated with androgen hormone agonist DHT (R1881) and antagonist enzalutamide (MDV3100). Then EVs were isolated from hormone-treated and untreated LNCaP cell culture medium. Then EVs were captured by biotinylated anti- CD9.C11 which was immobilized in a streptavidin-coated microtiter plate with EU^{+3}-coated nanoparticles (NPs) conjugated with lectins was used to detect the surface glycan of EVs. To maintain an equal amount of EVs in each well, 800ng total protein was added.

Results: Among twenty-four screened lectins, UEA (*Ulex europaeus* Agglutinin) presents a considerable amount of fluorescence signal to the EVs from the LNCaP cell line. We noticed that fucosylation in EVs-derived from LNCaP cell was increased by R1881 and decreased by MDV3100 which is indicated by the fucose-binding lectin UEA. Antibodies against tetraspanins were conjugates with NPs which was used for normalizing signal. Our results give a hint for androgen hormone and EVs glycosylation for detecting PCa from the simple assay.

Conclusion: Influence of glycosylation and androgen in PCa is a well-studied phenomenon. Therefore, identification of glycosylation in the surface of EVs from the simple lectin-NPs technique would be a useful approach for EVs-based cancer diagnosis.

Keywords: Prostate cancer (PCa), androgen hormone, extracellular vesicles (EVs), LNCaP cells, glycosylations.

ABBREVIATIONS

AR	Androgen receptor
ADT	Androgen diprivation therapy
BITC	Biotin isothiocyanate
BMI	Body mass index
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
CCV	Clathrin coated vesicles
DHT	Dihydrotesterone
DRE	Digital rectal exam
DMSO	Dimithyl sulfoxide
EDC	1-ethyl-3-(3`dimethylaminopropyl) carbodimide
EDTA	Eithylene-di-amine-tetraacitic acid
EVs	Extracellular vesicles
EM	Electron microscopy
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
FDA	Food and drug administration
mAb	Monoclonal antibody
MDV	Multivesicular bodies
mRNA	Messenger ribonucleic acid

MEV	Multivesicular endosome
NHS	N-hydroxysulfosuccinimide sodium salt
NPs	Nano-particles
PCa	Prostate cancer
PFA	Paraformaldehyde
PSA	Prostate specific antigen
PBS	Phosphate buffer saline
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma
TEM	Transmission electron microscopy
TRF	Time resolved fluorescence
TRUS	Transrectal ultrasonography
TSG101	Tumor susceptibility gene 101 protein
SEC	Size exclusion chromatography
LNCaP	Lymph node carcinoma of prostate
UI	Uranyl-oxalate
UA	Uranyl acetate

TABLE OF CONTENT

1. INTRODUCTION

1.1Prostate cancer

Prostate cancer (PCa) is considered as one of the common malignancies in male and second-highest death-causing cancers for males. Each year about one million new PCa cases are diagnosed worldwide (Jemal et al., 2011). As prostate cancer develops slowly in the patient's prostate, many patients die with prostate cancer rather than from the diseases. Still, the high mortality rate shows the severity of this cancer (Lukkarinen, 2002). Treatment of prostate cancer is easy but the diagnosis is difficult, as it has no symptoms in its early stage. Moreover, the current diagnostic method has a lack of specificity and sensitivity which allows the disease to develop into the metastatic stage. Lymph nodes are adjacent to the prostate gland metastasized in the beginning or in worse case to the bone, central nervous system, and other organs which are challenging to treat and cause death. Early and efficient diagnosis can save many PCa patients from sufferings. Therefore, it is essential to develop a sensitive assay involving non-invasive techniques, thereby reducing the need for unnecessary biopsies for prostate cancer patients.

1.1.1 Cellular architecture of prostate gland

The Prostate gland, shaped like an inverted triangle and the size of a chestnut, is an essential gland of the human body. The prostate gland is located between bladder and penis which secrete 30% of seminal fluid during ejaculation which acts as the nourishment for sperm. This exocrine gland can divide into four anatomical zones (McNeal, 1968; McNeal, 1980): transitional zone, central zone, peripheral zone, and anterior fibromuscular stroma. In the prostate gland, 25% of the volume is covered by the central zone of the gland whereas the transitional zone covers only 5%, but benign prostate hyperplasia occurs here. Peripheral zone covers most part of the prostate and prostate adenocarcinoma occurs in this zone. Rest of the prostate gland consists of fibromuscular stroma (McNeal, 1988).

Androgen regulates prostate cell growth and functional integrity by binding with androgen receptor (AR). Androgen is also an essential factor for treatment in PCa, because depletion

of androgen causes rapid prostate cellular apoptosis and atrophy (Huggins and Hodges, 1941).

1.1.2 Epidemiology of prostate cancer

Among all types of cancer, PCa diagnosed one of the highest numbers each year in males. In terms of mortality rate, PCa is placed after lung cancer for males. Mostly elderly persons, aged over 65, have a higher rate of PCa (Parkin et al., 2001). As it is a slow-growing cancer and diagnostic approaches for identifying PCa are still challenging, so many patients die with prostate cancer rather than from the complications of PCa (Sakr et al., 1993).

According to the global cancer statistics, the Australian and North American continents have the highest incidence rate of PCa, followed by Western, Northern and Caribbean countries. Asian countries have lower PCa incidence and mortality rate.

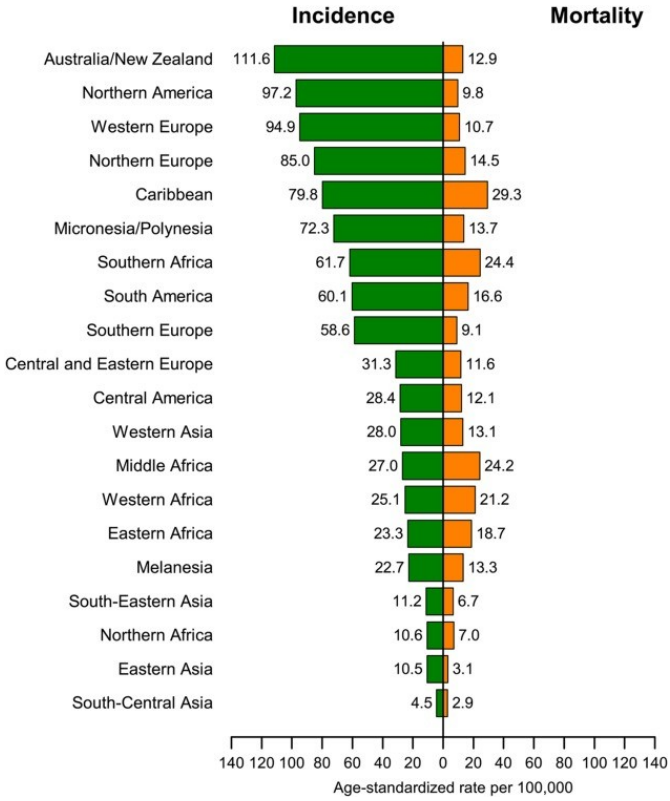


Figure 1: Incidence and mortality rate of PCa in different parts of the world (Torre et al, .2015)

Although in developed countries the number of PCa patients is higher, the mortality rate is higher in Caribbean countries. Because of early diagnosis and better treatment facility, the PCa mortality rate is lower in comparison with African and Caribbean countries.

Studies found that on an average 2.25 men die every day in Finland (Hass et al., 2008). According to the Finnish Cancer Registry in 2016, 888 men died from prostate cancer. The prostate cancer incidence rate is also growing every year in Finland.

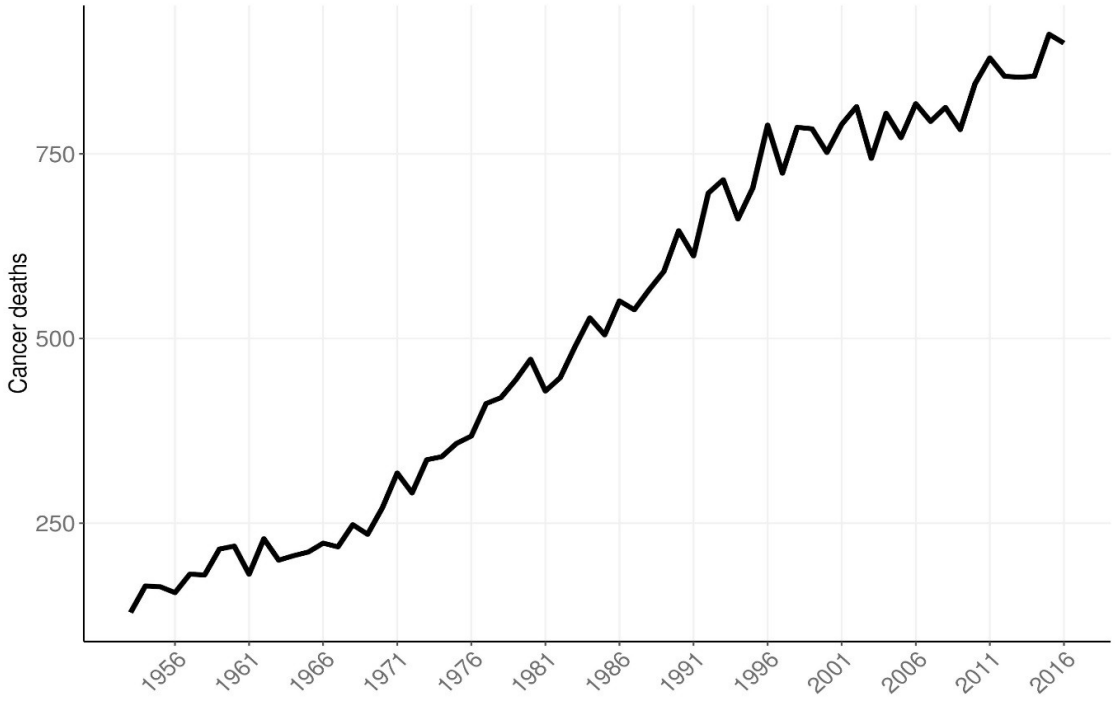


Figure 2: Prostate cancer deaths in Finland. (Finnish Cancer Registry)

1.1.3 Prostate cancer risk factors

Genetic and non-genetic factors initiate PCa and develop its progression. There are few factors which are considered as most important for PCa, such as age, ethnicity and family background with PCa.

Aging has a direct relation with PCa initiation. Age-specific studies show that the incidence curve rose after the age of 55 years and reached a peak in 74 years, the incidence rate

slightly declining after that (Gann, 2002). The family history of PCa can also be a factor for a large number of patients (Stanford and Ostrander, 2001). Another study showed that 42% risk from heredity for PCa where the study has taken place among 44,788 twins (Lichentenstein et al., 2000).

In comparison with Caucasian men, African men are at greater risk of PCa. The death rate of African men is two to three times higher (Chornokur et al., 2011). Other influential factors such as food habit, the steroid hormone, body mass index (BMI), lifestyle and environmental factors have an impact on the development of PCa.

The incidence of PCa in Finland also showed a similar pattern of aging-related PCa. From 1956 to 2016, there were more incidences among elderly people. Here PCa incidence starts at the age range of 50 years to 59 years. People from 70 years to 79 years are mostly diagnosed with PCa with a gradual rise in the curve.

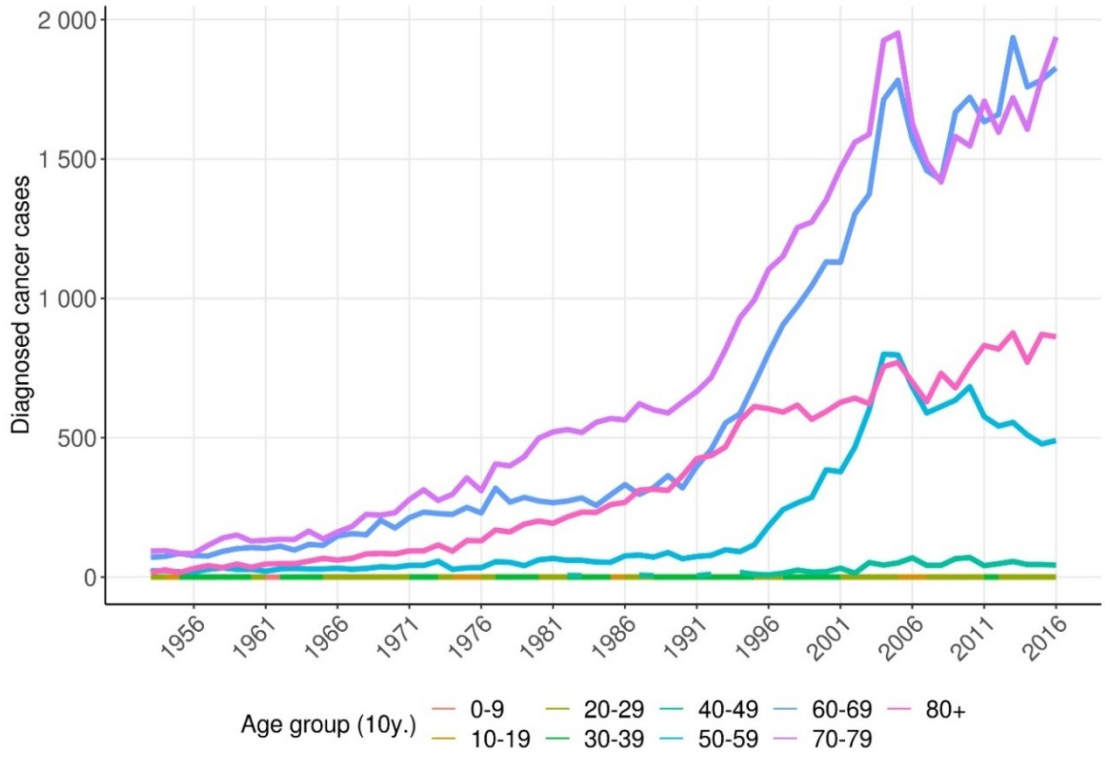


Figure 3: Age group dependent PCa incidence in 60 years from 1956 to 2016(Finnish Cancer Registry)

1.1.4 Prostate Cancer Progression

Abnormal or uncontrolled cell growth is considered as carcinoma. Genetic alternation or mutation of cell initiate cancer in the patient and multiple genetic changes create a situation for autonomous uncontrolled growth, metastasis, and angiogenesis.

The complexity of PCa involves several allelic losses which have been reported in different papers. Removal of the specific region of chromosome 8p was reported in 80% patients of PCa (Fujiwara et al., 1994; Chang et al., 1994; Matsuyama et al., 1994; Imbert et al., 1996; Wistuba et al., 1999). Different experiments show that removal of a certain region of chromosome locus 10q-23 guided to loss of PTEN (Phosphatase and Tensin Homolog) which is responsible for 50 to 80 percent of PCa cases (Trybus et al., 1996; Cher et al., 1996; Ittmann, 1996; Saric et al., 1999). In metastasized PCa, mutated PTEN is often detected (McMenamin et al., 1999). Genetic change or loss of whole PTEN increased proliferation and reduced apoptosis.

About 50% of PCa patients identified with gene alternated or mutated retinoblastoma (RB) gene which is a tumor controlling gene (Conney et al., 1996; Melamed et al., 1997). The progression of the metastatic stage of PCa occurred by the loss of the chromosomal 17p which include p53 gene (Cher et al., 1994; Brooks et al., 1996; Saric et al., 1999). Other studies showed that the development of metastatic subclones is associated with TP53 gene alternation (Hong et al., 2015).

Along with the changes in the tumor suppressor gene, other genetic variations occur in different cell cycle regulatory pathways. Androgen receptor signaling pathway gene alternation, mutation of cell cycle regulatory gene, gene alternation of apoptosis regulatory gene and telomerase activity regulatory gene variations also occur during PCa (Zhang et al., 1998; Shen and Abate-Shen, 2010).

1.1.5 Prostate cancer grading

PCa grading is one of the challenging tasks because of its heterogeneous character and multifocal nature. However, PCa grading is also important for finding the best available treatment option for patients. In 1950, TNM (tumor, node and metastasis) classification was introduced by physicians for cancer grading. In the TNM classification, T stands for tumor size (T1 to T4), N for the number of nodes involvement (N0 to N1) and M for the metastasis (M0 to M1). In 1997, a revised TNM grading system was given by The American Joint Committee on Cancer (AJCC).

T (Primary Tumor Size and Involvement)	**T1**	The existence of the tumors are discovered, but they are not detectable by imaging
	T2	Detectable tumors are confined within a single lobe (T2a) or both lobes (T2b) of the prostate
	T3	Tumors have extended through the prostate capsule (T3a) and also might have invaded the seminal vesicles (T3b)
	T4	Tumors have invaded the adjacent organs other than seminal vesicles
N (Lymph node metastasis)	**N0**	Tumors have no metastasis in a lymph node
	N1	Tumors have regional metastasis in lymph node
M (Metastasis)	**M0**	Tumors have no metastasis on the remote parts of the body
	M1	Tumors have remote metastasis anywhere in non-regional lymph nodes (M1a), bones (M1b) or other sites (M1c)

For histopathological inspection, The Gleason grading system is trusted and known for PCa grading. This grading method is used to predict the behavior of cancer progression. The Gleason score is graded in two influential patterns which appear on a range from 1 to 5, whereas Gleason score 5 is considered as the most aggressive. Gleason score grading 2 to 4 is considered as well-differentiated cancer, whereas score 5 to 7 and 8 to 10 are considered as moderately differentiated and poorly differentiated cancer, respectively.

Usually well-differentiated cancer progresses slowly, so it is possible to identify from poorly-differentiated, aggressive cancer but difficult to distinguish from moderately differentiated cancer. However, the Gleason grading system has some drawbacks due to intra and inter-observer variability and under-estimation of the tumor grade in 33-45% of cases (Bostwick, 1994).

1.1.6 Current Screening methods of PCa

Detection of PCa in the early stage can substantially decrease the mortality. Currently, there are different screening methods available for PCa detection for the patients.

Digital rectal exam (DRE) was earlier one of the earliest screening methods for PCa. During a routine checkup in hospital, most of the PCa patients came to know about their disease condition. However, DRE is not useful for detecting local and asymptomatic cancers. It was found that the DRE screening method had considerable variability of detecting PCa ranging from 0.1% to 25% patients, but it was found that cancers per positive biopsy ranging from 6% to 50% (Brawer, 2000).

Another technique for prostate cancer screening is Transrectal Ultrasonography (TRUS). TRUS is used for prostate imaging. For PCa screening, TRUS-guided needle biopsy is considered as the gold standard. However, several studies showed that TRUS-guided biopsy had the chance of false negative results up to 10 to 30% and often missed to identify PCa (Stewart et al., 2001; Patel et al., 2004; Barqawi et al., 2011).

Prostate-specific antigen (PSA) measurement is another popular screening method for PCa. The PSA screening method was approved in 1994 by the FDA. PSA measurement is more effective than DRE or TRUS for early detection of PCa. PSA also has its drawbacks; it often gives false positive result without any malignancy. Mainly because of BPH (Benign Prostate Hyperplasia) or prostatitis, the level of PSA value elevated. Among all current diagnostic approaches, PSA is still considered as the most sensitive test for PCa screening, and it reduces a substantial amount of PCa mortality (Schroder et al., 2014). However, it is controversial to overuse the PSA test for population leads to unnecessary biopsies with a false positive result (McShane et al., 2006). PSA test along with TRUS gives better diagnosis precision, and is recommended by physicians.

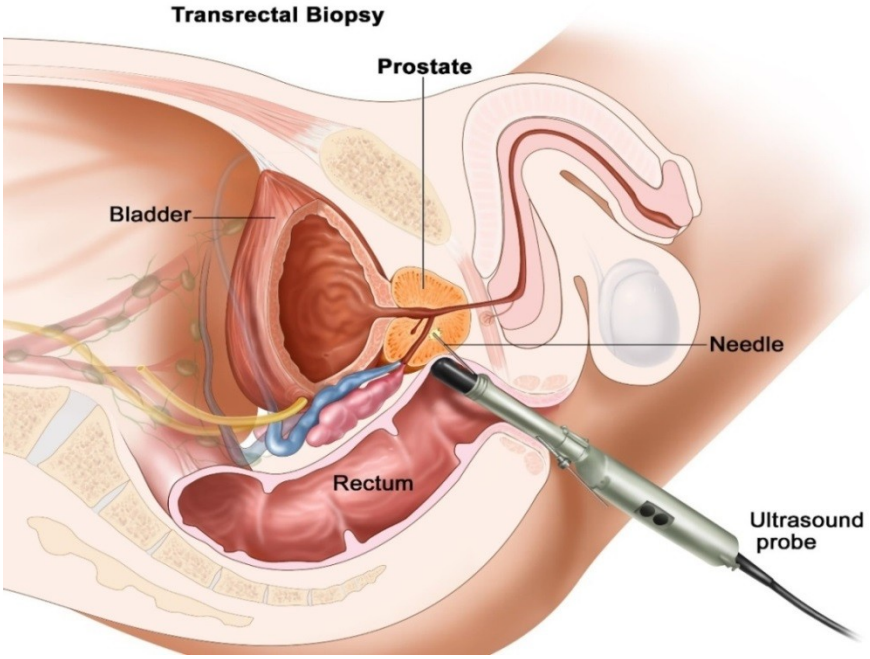


Figure 4: Transrectal Ultrasound guided needle biopsy. (Illustration taken from © 2005 Terese Winslow)

1.1.7 Future PCa diagnostic approach

Although the PSA test showed a better result than other existing diagnostic approaches, but the absence of sensitivity and specificity from current diagnostic methods creates an opportunity for a new diagnostic approach. A new diagnostic approach will reduce

unnecessary biopsies for prostate cancer. Recent studies show that extracellular vesicles (EVs), which are released by the cells and available in all body fluids, could be an option for identifying tissue-specific markers (Duijvesz et al., 2015).

EVs glycosylation changes are a common phenomenon in cancer, so that EVs can provide information about the condition and alternations in the metabolism of the host cell. Altered glycans are found on the surface of tumor cells and have great potential in cancer diagnosis. Altered glycans identification in the EVs have the possibility to be a viable diagnostic target for cancer (Satomaa et al., 2009; Gerlach et al., 2017). The PCa cell line derived-EVs detection with a prostate-specific marker will be a new perspective for the diagnosis of PCa (Minciacchi et al., 2017). An effective diagnostic approach for the prostate cancer can save millions of lives by early detection.

1.2 Extracellular Vesicles

1.2.1 EVs subtype

The EVs are mostly described as exosomes which comprise vesicles of endosomal origin (50-150 nm) and outer membrane-derived vesicles called microvesicles (200-1000 nm) (Harding, Heuser and Stahl 1983; Muralidharan-Chari et al., 2009). The EVs are produced directly from the cells. The EVs can be collected and isolated with different methods from the bodily fluid. Approximately 9,769 proteins have been identified from the EVs (Keerthikumar et al., 2015).

The exosomes are lipid bilayer EVs which are ranging from 30 to 40 nm for upper diameter and 100 to 130 nm for lower diameter. The exosomes are spherical in shape and secreted from multivesicular bodies (MDV) (Johnstone et al., 1987; Rapso et al., 1996; Théry et al., 2006; Théry et al., 2009; Conde-Vancells et al., 2010). The EVs mainly contain a large amount of proteins, lipids and nucleic acids which have an essential role in intercellular communication (Théry, 2011).

The microvesicles are other types of EVs which are of a more complex nature than exosomes (Muralidharan-Chari et al., 2009). The microvesicles have a diameter ranging from 200 to 1000 nm and a round shaped structure (Marzecsco et al., 2005; Booth et al., 2006; Hara et al., 2010).

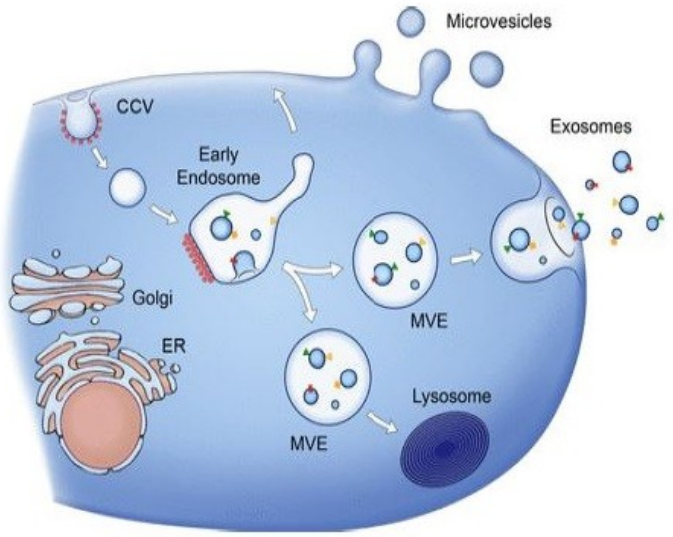


Figure 5: Production and release of EVs. CCV=clathrin coated vesicles; MVE=multi-vesicular endosome;ER=endoplasmic reticulum.(Rapso et al., 2013)

Another category of EVs are apoptotic bodies which have a size range between 500 nm and 2000 nm (Akser et al., 2013; Théry, Ostrowski and Segura, 2009). The apoptotic bodies mainly consist of cytosol, endoplasmic reticulum and nucleus (histone, condensed chromatin) (Cline et al., 2004; Dieker and Muller, 2009). The apoptotic bodies have higher amounts of total protein contents but lower protein variability compared to exosomes from thymic T cells (Turiak et al., 2011).

1.2.2 Biogenesis of EVs

The biogenesis of EVs is still not properly understood. The microvesicles formation happens with outward budding of the cell membranes which is associated with the flipping of phosphatidylserine to the outer membrane leaflet (Hugel et al., 2005; Cocucci and Meldolesi, 2015). Tumor susceptibility gene-101 protein (TSG101) and ESCRT-III are

involved in the releasing of microvesicles (Coccuci and Meldolesi, 2015). The EVs are generated from inward budding of the surface membrane and form a preliminary endosome with cell surface protein (Pan et al., 1985; Harding et al., 1983). A large number of tetraspanins are found in the EVs from the β cells (Escola et al., 1998). The tetraspanin 8 over-expressions result in the release of the EVs from the rat pancreatic adenocarcinoma cells (Rana et al., 2012; Nazarenko et al., 2010). The ESCRT complex works as the best sorting machinery for approximately thirty proteins which are divided into four groups (Hurley 2015; Kowal et al., 2014). All these four groups are involved in different interactions, the active inward budding of endosomal membranes are done by ESCRT-I and II (Villarroya-Beltri et al., 2013).

1.2.3 Bio-molecular content of EVs

In recent studies, the biomolecular contents of EVs have caught attention of the scientific community and those are now studied in different profiling technologies such as proteomics, transcriptomics, lipidomics, and glycomics.

Transmembrane and cytosolic proteins' diversity and specificity have been studied in proteomics. Post-translation modifications such as phosphorylation and glycosylation are also studied in detail. Most common proteins found in the EVs are small GTPase (Rab Proteins), heat-shock proteins (HSP70, HSP90), tetraspanins (CD9, CD63, CD81 and CD82), endosomal proteins (LAMP-1, flottillin-1), and ESCRT components with accessory proteins (ALIX, TSG101) (Raposo and Stoorvogel, 2013; Colombo et al., 2014; Mathivanan et al., 2010). A significant amount of bioactive proteins such as transcription factors, enzymes, chaperones, signaling molecules, ion channels and transporters which represent their parents' cells are also found in the EVs (Colombo et al., 2014).

The nucleic acid is another bio-molecular content found in the EVs. Mostly mRNA and miRNA are available but different non-coding RNAs are also found in EVs (Valadi et al., 2007; Miranda et al., 2014; Ratajezak et al., 2006). Recent studies on miRNA content of the EVs had showed the possibility to work as a biomarker (Mittelbrunn et al., 2011; Ohshima et al., 2010; Miranda et al., 2010; Sole et al., 2015).

The lipid content of the EVs is less studied in comparison with protein and nucleic acids. The exosomal lipids have a link with lipid raft areas (Trajkovic et al., 2008). Sphingomyelin and cholesterol are both common in the EVs and cell surface (Llorente et al., 2013). In other studies, the prostate cancer cell EVs and renal cell carcinoma EVs have different variations of glycosphingolipids (Llorente et al., 2013; Del Boccio et al., 2012).

Carbohydrate is another, less studied but important bio-molecular molecules. Lectin microarray (LM) approach for surface glycan profiling of EVs from H9 T cell line founds N-glycans, N-acetyl-lactosamine, (α2 to 3) and (α2 to 6) sialic acid (Neu5Ac or NeuAc), fucose (Fuc.) and mannose (Krishnamoorthy et al., 2009). EVs from human skin cancer cells are (SkMel-5) enriched with poly-N-acetyl-lactosamine, high mannose,(α2 to 6) Neu5Ac (Batista et al., 2011).

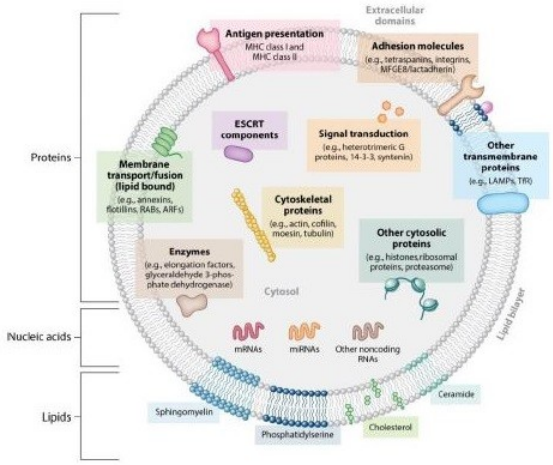


Figure 6: Bio-molecular content of extracellular vesicles **(**Colombo et al. Annu Rev Cell Dev Biol. 2014; 30: 255-289)

1.2.4 Functions of EVs

The EVs are well known for their intercellular communication (Valadi et al., 2007). Different types of cells release EVs which influence nearby microenvironment as well as distant parts of the body using blood or other biological fluids as carrier (Luga et al., 2012; Lai et al., 2010; Salido-Guadarrama et al., 2014). In different studies, it has been described

that the EVs work as a carriers of active bio-molecules such as mRNA, miRNA, receptors, and enzymes (Valadi et al., 2007; Hakulinen et al., 2008; Pan et al., 1985).

The EVs have a functional effect for tumorigenesis (Azmi et al., 2013; Brinton et al., 2014). Breast, colon and pancreatic cancer cell lines released a high level of the EVs which contain protein such as miRNA, MHC I, MHC II, FasL, TRAIL, NKG2D ligand and TGF-β (Simona et al., 2013; Mincheva-Nilsson and Baranov, 2014; Zhang et al., 2015). FasL released from tumor cells EVs have affect on apoptosis of $CD8^{+}$T cells which leading to immune suppression (Wieckowski et al., 2009; Kim et al., 2005). The tumor-cell derived EVs have shown immunosuppressive tumor niche through the transfer of TGFβ, modulation of tumor-associated fibroblasts and generation of angiogenesis (Webber et al., 2010; Park et al., 2010; Webber et al., 2015).

1.2.5 Isolation and storage procedure for EVs

The isolation of EVs from biological fluid and cell culture medium can be done with different methods. During the isolation procedure, there are a number of important factors involved such as the source of biological fluid, the degree of purity, sample volume and number, downstream analysis application, processing time and equipment, specific EVs subtype (Tauro et al., 2012). Among all isolation techniques, the most popular EVs isolation methods are given below.

- Ultracentrifugation
- Size exclusion chromatography
- kit-based precipitation
- Ultrafiltration

Ultracentrifugation is the most common EVs isolation technique from the biological fluid. In this method, centrifuge speed is 100,000× g (ultracentrifugation) for separation of the EVs pellet (Fernández-Llama et al., 2010; Théry et al., 2006). Ultracentrifugation method efficiency depends on a few factors such as the viscosity of the sample, rotor speed and time (Cvjetkovic, et al., 2014; Fatemeh et al., 2012). The isolation of the EVs from the cell culture medium needs 100,000×g for 90 minutes, whereas urinary EVs need 200,000×g for

150 minutes (H. Zhou et al., 2006; Chen et al., 2013; Hogan et al., 2014;). The ultracentrifugation isolation method in combination with sucrose density gradient centrifugation produces high quality EVs (Hogan et al., 2014). The ultracentrifugation method has some drawbacks such as expensive equipments, time-consuming and unsuitable for large volume sample (Hogan et al., 2014; Wang and Wei, 2014; Van Deun et al., 2014). Despite of the limitations, this method is still considered as the gold standard for the EVs Isolation.

Size-exclusion chromatography (SEC) is another technique for the Isolation of EVs. The easy separations of lipoproteins and proteins from the EVs are the advantage of using SEC. The SEC and the ultracentrifugation method produce similar isolation result from cell culture supernatant (Lobb et al., 2015; Nordin et al., 2015). Initial concentration steps and collection of EVs fractions are tedious and problematic. Thus, this method of isolation is not widely adopted by the researchers.

Ultrafiltration has been widely used as an isolation method for the EVs. Commercially available 3K to 100K Da (MWCO) centrifugal filters devices are used for isolation in this method (Lobb et al., 2015; Cheruvanky et al., 2007). The main advantages of the ultrafiltration method are shorter isolation period, low cost, easy to use and suitable for large volume of sample (Cheruvanky et al., 2007). The ultrafiltration also has some drawbacks such as aggregation of the EVs, loss of the smaller EVs, low sample quality (Gerlach et al., 2013; Lobb et al., 2015; Merchant et al., 2010; Cheruvanky et al., 2007; Rood et al., 2010).

There are a number of EVs isolation kits available in the market. Total EVs Isolation (Invitrogen), ExoQuick (System Bioscience), Exo-spin (Cell Guidance System) are widely used isolation kit. The efficiency and purity of these isolation kits are very low (Van Deun et al., 2014; Zubiri et al., 2013; Lobb et al., 2015).

1.2.6 Glycosylation of EVs

The glycosylation is an essential biological event in the EVs. The diversity of glycosylation arises from the glycosidic linkage (Pinho and Reis, 2015). Glycosylation is the result of the gene expressions and functions of many glycosyltransferase that add other carbohydrate sugar moieties, proteins or lipids for creating different glycoconjugates.

Glycan profiling technologies and glycosylation of the EVs create the opportunity for the discovery of biomarkers for the PCa. The glycans are the bio-molecular units having various linkages to different macromolecules such as lipid and proteins. The glycosylation of proteins are usually via covalently attaching carbohydrate moieties on the backbone of polypeptides. The attachment of carbohydrate to polypeptides can be O-linkage to serine/threonine (Ser/Thr) or N-linkage to asparagines (Asp) (Cummings, 2009). Tumor-associated specific glycosylation alternations are mainly sialylation, N-linked glycan branching, O-glycan truncation. The tumor-associated glycans were usually with an elevated level of overall sialylation (Kannagi et al., 2008).

The glycosylation changes are common phenomenon in cancer. Glycosylation of EVs provides information about the condition and alterations in the metabolism of the host cell. Altered glycans are found on the surface of the tumor cells and have a great potential in cancer diagnosis.

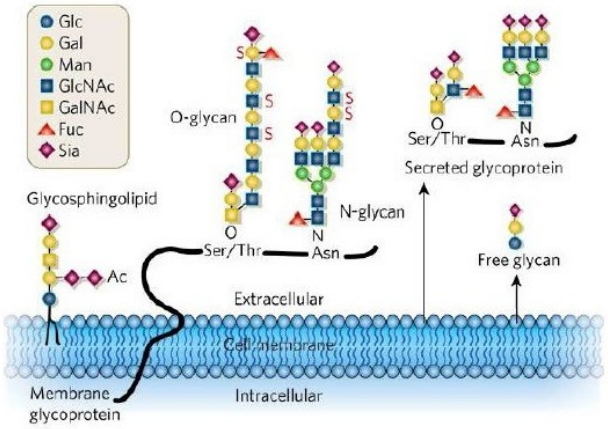


Figure 7: Illustration of the basic subtypes of the surface-bound and secreted carbohydrate structures associated with the mammalian cells. (Varki, 2007)

1.3 Androgen hormone

During the PCa progression, steroid hormone plays an important role through the androgen receptor (AR) (Munkley et al., 2016). The PCa cell growth, proliferation, and invasion are dependent on the AR (Hara et al., 2008; Snoek et al., 2009). The androgen-hormone regulates the process of glycosylation by influencing the glycosylation enzymes. The androgen-hormone mainly works on the gene expression of enzymes which involves multiple steps of glycosylation synthesis (Munkley et al., 2016).

A set of eight androgen-hormone regulates glycosylation genes are essential for the PCa progression. These androgen-hormones regulates by four different synthetic pathways. The pathways are O-glycan, chondroitin sulphate, N-glycan, and HBP (Munkley et al., 2016). At the beginning of the cellular transformation, O-glycans are changed and plays a vital role in tumor progression (Pinho and Reis, 2015). During the PCa, elevated O-GlcNAc is common which is created by HBP pathway (Kamigaito et al., 2014). Androgen regulates the pathway of producing chondroitin sulphate and is elevated in amount during PCa (Ricciardelli et al., 1997; Gandhi and Mancera, 2008; Ricciardelli et al., 2009). ST6GAL1 and EDEM3 enzymes are linked in N-glycan processing which is controlled by the androgen-hormone. The sialylation of N-glycans are controlled by ST6Gal1 and its over-expression has a direct link with the various types of tumor (Swindall et al., 2013)

The secretion and expression of CD9 in the EVs are increased by the treatment with the AR (androgen receptor) agonist R1881 (Chuan et al., 2005). Altered glycosylation in the PCa via AR might change some of these biological processes. The prostate gland usually secrets a considerable amount of glycoproteins, thus the altered glycans have the possibility for becoming glycovariant biomarkers for the PCa.

1.4 Lectin and their applications to glycoanalysis

The lectins are carbohydrate-binding proteins. Usually lectins are binding to glycoproteins by targeting the glycan moiety. The lectins have frequently been used as tools for studying glycosylation and can assist in cancer diagnosis. The lectins are mostly bind specifically

with glycoconjugates and can be used to differentiate between normal and cancerous individuals (Mody et al., 1995).

In order to practically benefit from the lectin-glycan interaction, assay constructs of highly improved performance are needed where the binding strength of the lectins is highly enhanced without negatively affecting the binding specificity. In earlier studies, it was found that Europium (Eu^{3+})-nanoparticles (NPs) aided approach could be greatly enhanced the binding strength of lectins (Gidwani et al., 2016; Kekki et al., 2016).

The lectin-NPs aided approach open possibilities to discriminate between glycostructures which differently expressed in the PCa; especially its more aggressive forms vs. non-cancerous forms. Our group has developed highly sensitive and non-invasive time-resolved fluorescence (TRF)-immunoassay to capture and detect the EVs from the LNCaP cell culture medium. With this advanced method, we aim to identify the glycosylation patterns of the EVs isolated from androgen-hormone treated PCa cell lines. For identification of the glycosylation in EVs, we proposed the electron microscopy which will help us for the visualize and capture images.

2. OBJECTIVES

The PCa diagnosis from the PSA test and needle biopsy had specificity and sensitivity problems. From the previous works, we came to know that lectin coated Eu^{3+}-nanoparticles approach can help in differentiating the samples by selectively binding to the cancerous-CA125 (cancer antigen 125) (Gidwani et al., 2016). Based on this information, we hypothesized that this lectin-NPs approach can be used to the identification of the glycosylations affected by the hormone-regulated process. Thus, discrimination between androgen-sensitive and insensitive EVs through glycosylation profiling would be an attractive option for diagnosis for the future. Therefore, the aim of our thesis project is given below.

I. Lectin coated Eu^{3+}-nanoparticle assisted surface glycosylation profiling of the EVs.
II. Identify the glycosylation changes on the surface of the EVs by inducing and blocking of androgen receptor (AR) pathways.
III. Finding a novel lectin glycovarient biomarker for diagnosis of PCa.

3. MATERIALS AND METHODS

3.1 LNCaP Cell Culture

3.1.1 LNCaP Cell thawing and medium preparation

Androgen-sensitive LNCaP cells are prostate adenocarcinoma cells which are used as PCa cell line in this project. LNCaP cells were preserved in liquid nitrogen (temperature -200℃). The cryogenic vial of LNCaP cells was taken from the tank of nitrogen and kept in a box with dry ice before using it. Cell culture medium RPMI-1640(GIBCO-L Glutamine, Thermofisher scientific, and USA) was heated to 37℃. Then we used 10% FBS (fetal bovine serum) (GIBCO, USA). Cell culture facility was maintained with GMP guideline and ethanol was used everywhere to maintain cleanliness.

All necessary glassware such as an electronic pipette, flask for cell culture, 10 mL serological pipette and sterile paper, were put inside the laminar hood. Cryogenic vial was put halfway in the water bath for 1 minute and moved around. After that, the cells were transferred immediately into a falcon tube and 5 mL of cell culture medium was added. Centrifugation of 1000 rpm for 5 minutes suspended the cell at the bottom. Then the rest of the DMSO (Dimethyl sulfoxide)-contained medium was discarded from the cryogenic vial. 10mL of medium was added in the tube and a pipette was used for mixing the cells in the medium. Then the medium was transferred into the culture flask and checked with an inverted bright field light microscope. The cell culture flask was placed inside the incubator for 37.4℃ and 5% of CO_2.

3.1.2 Cell growth observe, confluency and changing medium

Cell growth observation is important for the cell culture process. The culture flask was checked with as inverted light microscope and the confluency was checked to determine the number of the cells in the flask. After a few days, the medium color changed from pink to colorless or yellowish, the cell culture medium was changed then. When the cells reached the 80% confluency in the flask, the old flask was changed into a new flask.

3.1.3 LNCaP Subculture

Confluency of LNCaP cells was needed to be more than 80% for subculture. LNCaP cells are adherence cells which grow after binding with the flask floor. Thus, to detach the cells from the floor, we used RPMI-trypsin/EDTA solution; 9 mL RPMI-1640 and 1mL of 1x trypsin/EDTA (Eithylene-di-amine-tetraacitic acid) were added in a tube. LNCaP cell culture medium was removed to a falcon tube (waste) by decanting of pipetting. The 10 mL RPMI-trypsin solution was added and the flask was incubated at 37.4 ℃ and 5% of CO_2 for 15 minutes. Then the flask was pipetted with RPMI-trypsin until the cells detached from the bottom. The medium was transferred into a new tube and centrifuged for 1000 rpm for 5 minutes. After discarding the supernatant, pelleted cells were resuspended into the medium by pipetting. Then the solution was transferred into two new T75 flasks (2.5mL) (Thermofisher). Cells were observed and put into the incubator.

3.1.4 Androgen receptor agonist and antagonist treatment of cell culture

Then cell culture mediums were treated with metribolone (R1881) and enzalutamide (MDV3100). The 10 nM of R1881 was added with the cell culture media. In the other flask, 10 mM androgen receptor antagonist enzalutamide (MDV3100) was added. Then the two flasks were kept in the incubator for growth. When the confluency reached about 70 to 80% and then the cell culture medium was collated for the EVs isolation.

3.2 Biotinylation of antibodies

Biotinylation is a process by which covalent bond happened between biotin and protein, nucleic acid or other molecules. The functional group of different specificity and different solubility characteristics are very important for biotinylation agent to determine specific macromolecule and cell surface. Biotinylation of monoclonal antibodies (mAbs) anti-CD9

(R&D, Abingdon, UK), antigen binding fragment antibodies (mAbs) CD9.C11 (developed in Kim Pettersson Lab) has been done following the protocol discussed below.

Biotinylation was done with the slight modification of the given protocol by Väisänen et al., 2006. The antibody was checked whether it contains any preservative or not; if it contains the azide buffer (known as preservative) then we need to remove the buffer from the antibody. The biotin isothiocyanate (BITC) was weighted (MW=404g/mol) in an Eppendorf tube and was dissolved in EtOH to make of 10mM (e.g 0.001g/254 µl). The solution was spin down at 1500 rpm to remove the non dissolved impurities. The supernatant was used for reaction. The 3mL glass bottle was used for the reaction and the pH of the antibody solution was adjusted with 1/10 volume of 0.5 M carbonate buffer, pH 9.8 and mixed well. The BITC amount was calculated 40 fold excess of biotin and then the solution was added into protein. The solution was kept in incubation for 4 hours at room temperature.

During the incubation time, the gel filtration column was equilibrated with TSA buffer. The unreacted BITC was removed from the solution by NAP-10 (GE-Illustratra, Diegem, Belgium) gel filtration column. The concentration was measured with Bio-Rad Bradford Assay and biotinylation degree was determined. The DPTA purified BSA was mixed to a concentration of 0.1% and then the biotinylated antibody was stored at +4º C.

3.3 Bradford assay

Bradford assay determines the concentration of the solubilized protein. The biotinylated antibody had been used for the Bradford assay to determine the protein concentration. The 10 µl microtiter well (Maxisorp) of IgG was pipette as follows: blank, 0.5 mg/mL, 0.4 mg/mL, 0.3 mg/mL, 0.2 mg/mL, 0.1 mg/mL, and 0.05mg/mL in three replicas. The 10 µl of sample was pipette in three replicas.

The Bradford reagent was mixed with the distilled water of 1:4 concentrations and then the solution was filtered through 0.8 µm filter paper. The 200 µl of Bradford reagent was added in each well. The SA plate was shacked for 1 minute in room temperature. After that the absorbance was measured in Victor machine (Delfia measurement).

3.4 Biotinylation degree measurement

The percentage of the protein that binds with biotin was determined by biotinylation degree measurement. The biotinylation degree measurement method of Der-Balin et al. 1990 followed with slight modification. A streptavidin-coated microtiter strips (SA strips) was pipette with the biotinylated antibody 50 ng/200 µl in assay buffer (AB) of each well and then made three replicates. The plate was shacked in room temperature for 1 hour. The anti-mouse IgG coated (RAM strips) was pipette with biotinylated antibody of different dilution as 20 ng, 10 ng, 5 ng, 2.5 ng, 1.25 ng, 0.65 ng/200 µl assay buffer for each well in three replicates for the standard.

The sample was taken as 100µl/well and 100 µl assay buffer for three replicates. The plate was shaken for two hours in room temperature and was washed 2X. Anti-mouse IgG-Eu 100ng/200µl per well in AB was filtered through 0.22 µm filter. Then the SA plate was shaken for two hours in room temperature and was washed 6X. The enhancement solution 200µl per well was added and was shaken the plate for 10 minutes. Europium signal was measured by Victor (Delfia measurement).

3.5 Activation of Nanoparticles

The activation of nanoparticles and labeling of the lectins had two important parts. The protocol of Härma et al., 2001 for nanoparticles activation was followed here. The nanoparticles (Seradyn Indianapolis IN, USA) were mono-dispersed and carboxyl-modified fluoro-max polystyrene beads. The lectins (Table 1) and anti-CD9 antibody were coupled co-valently with nanoparticles (Malaspina et al., 2017, Gidwani et al., 2016). The Europium nanoparticles (2×44,89of 95 nm, Fluro-max dyed carboxylate-modified Microparticles, 2.22E10/µl) were applied to 1 nanosep 300 kDa omega centrifugal device (corresponds to 1E12 particles respectively) for 7000 rpm, 5 minutes. The 200 µl of conjugation buffer was added. Tip sonication (65% amplitude, 0.5 cycles, and 30 pulses) was performed to resuspend the particles. The particles were transferred to 1.5mL eppendorf tube. NHS (N-

hydroxysulfosuccinimide sodium salt) and EDC (1-ethyl-3-(3`dimethylaminopropyl) carbodimide) were added to one tube at a time. The tube was vortex in the room temperature for 15 minutes. The solution was divided into two 1.5 mL of eppendorf-tube.

3.6 Lectin labeling with activated Eu^{3+}-nanoparticle

The lectin labeling was done according to the publication of Von Lode P. et al. 2003. The coating solution was made with 100 µg of lectin, conjugation buffer and 100 mM NaCl. The nanoparticles solution and lectin solution were mixed and incubated on shaking machine for 30 minutes. The pH was raised to 8.0 by adding 43 µl of 1M carbonate buffer for every mL of reaction and was incubated on vortex for 30 minutes. The 1% BSA was added to block the remaining active sites. The solution was kept in 4°C rotary mixture.

The mixture was purified by 1 nanosep 300 KDa and centrifugation at 8000 rpm for 5 minutes. The 150 µl of storage buffer was added on the filter. The mixture was sonicated and vortexed. Then 0.2% BSA was added from 20% BSA stock to block the rest of the active sites. The mixture was kept in 4°C freezer for a few days to settle down the aggregates. After that the nanoparticles were vortexed and centrifuged at 2000 rpm to remove the aggregate. The particles concertation was measured. Our lectin coated Eu^{3+}-nanoparticles kept in the fridge before use.

Lectin List with their full name and their major carbohydrate binding specificities is given below.

S. No	Lectin	Full name of Lectin	Major Carbohydrate binding specificities
1	MAA II	*Maackiaamurensis* agglutinin II	α2-3-linked sialicacids
2	AAL	*Aleuria aurantia* lectin	α1-6Fuc
3	UEA	*Ulex europaeus* agglutinin	Fucα1-2Gal
4	WGA	Wheat germ agglutinin	Terminal *N*-acetylglucosamine or chitobiose
5	WFA	*Wisteria floribunda* agglutinin	GalNAcα or β- 3 or 6 position of galactose
6	TJA-II	*Trichosanthes japonica* agglutinin	Fucα 1-2Gal and β-GalNAc

7	DSL	*Datura Stramonium*Lectin	(β-1,4) linked N-acetyl glucosamineoligomers
8	MGL	Macrophagegalactose-type lectin	Terminal α-or β-linked GalNAc
9	DC SIGN	Dendritic cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN)	Nonsialylated Lewis antigens and high mannose-type structures
10	MBL	Mannose binding lectin	Fucose, mannose/mannan
11	FCN 1	Ficoline 1	N- acetylglucosamine (GlcNAc), mannan
12	FCN 2	Ficoline 2	N- acetylglucosamine (GlcNAc),mannan
13	FCN 3	Ficoline 3	N- acetylglucosamine (GlcNAc),mannan
14	Galect in-3	β-galactoside binding lectins	galactomannans, mannan
15	Galect in-4	β-galactoside binding lectins	SO_3->3Galβ1->3GalNAc pyranoside
16	VVL	VIcia Villosa Lectin	N- Acetylgalactosamine
17	BPL	Bauhinia Purpurea Lectin	N- Acetylgalactosamine, Lactose
18	CLEC 7A	Dectin 1	β -1,3- linked and β-1,6- linked glucan
19	CLEC 6A	Dectin 2	High mannose carbohy
20	MMR	MannoseReceptor	Mannose, N- Acetylgalactosamine, Fucose residues on glycans

3.7 Immunoassay

Immunoassay determines concentration of the cellular small molecule in solution by binding with the antibody. In our project biotinylated antibodies of CD63, CD9, and CD9.C11 were used as the capture, and 24 different lectins coated with Eu^{3+}- nanoparticles were used as the tracer. The streptavidin plate (KaiSA96, Kaivogen, Turku, Finland) was prewashed with 1X DELFIA plate washer (Perkin-Elmer).

The biotinylated antibody with assay buffer (Kaivogen, Turku, Finland) was given as 200ng/30µl per well. The plate was incubated in room temperature and 2X washed after one hour. The isolated EVs of LNCaP cell culture medium were added as the sample in different concentration per well with assay buffer. One hour of shaking of SA plate at 600 rpm and the plate was washed 2X in DELFIA plate washer.

The lectin coated Eu^{3+}-nanoparticles were added with assay buffer at a concentration of 1E7 per well per 30 µl. The SA plate was incubated in room temperature with shaking at 600 rpm for one hour. Then 4X wash of streptavidin plate was done and put the plate for measuring the signal in Victor^{TM}1420 multilabel counter (Perkin-Elmer). Europium from surface program gave the binding fluorescence signals of EVs.

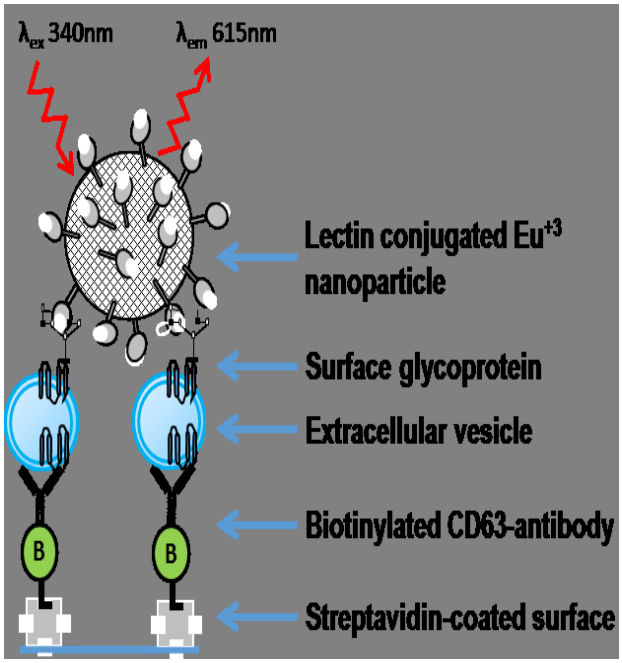


Figure 8: Nanoparticle-based time resolved-fluorescence immunoassay. The captured EVs are detected with Eu^{+3}-nanoparticles coated with lectins.

3.8 Extracellular vesicles isolation

3.8.1 Isolation methods

There were different isolation methods available from the cell culture media. Ultracentrifugation, size exclusion chromatography method and centrifugal ultrafiltration were used in our research.

3.8.2 Ultracentrifugation

Differential ultracentrifugation was considered as the gold standard for the EVs isolation. We had followed the protocol based on Kowel et al., 2016 with slight modification. The cell culture medium of 70 to 80% confluency was collected for the isolation. The cell culture mediums were treated with androgen hormone agonist (R1881) and androgen hormone antagonist (MDV3100).

The medium was centrifuged for 10 minutes at 300×g for 4°C. The supernatant were collected and removed the cells from the tube. The collected supernatant was centrifuged for 2000 ×g for 4°C for 20 minutes. Then suspended apoptotic bodies (200nm-5µm) were removed and was collected the supernatant for further centrifugation.

The supernatant was collected in a special tube (Beckman Coulter tube) and centrifuged for 45 minutes with 10,000×g in 4°C to suspend the microvesicles (100-800 nm). The supernatant was collected from the tube carefully and centrifuged for 100,000×g (Beckman Coulter Ultracentrifuges) for 4°C for 90 minutes. The layers of suspended EVs (30-150 nm) were collected. Then PBS was added to resuspend and passed through a 0.22 µm filter and 100 µl PBS was added to dilute the solution.

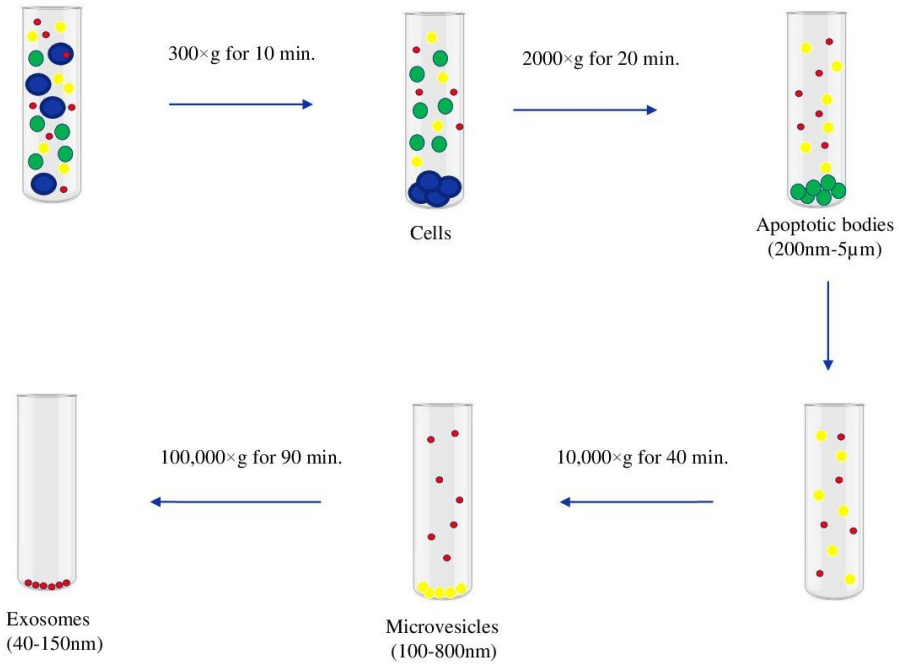


Figure 9: A schematic diagram of differential ultracentrifugation.

3.8.3 Size exclusion chromatography

Size exclusion chromatography was another popular method of isolation of EVs. Size exclusion chromatography kits (qEV single, Izon Science) were used to separate EVs from the cell culture medium. The larger molecule has crossed the pores but smaller molecules trapped in the pores of the size exclusion chromatography (Lobb et al., 2017, Anita et al., 2014).

The medium was centrifuged at 3000×g for removing the cells and small protein fractions from the medium. The supernatant was collected and rinsed the column with PBS. The 100 µl of cell culture medium was pipette on to the column. Then more PBS was added in the column to elute EVs. The fraction was collected from the column.

3.8.4 Ultrafiltration

Another popular method for EVs isolation was ultrafiltration. The centrifugal ultrafiltration method allowed the only specified size of microvesicles from the liquid and EVs were separated. In the ultrafiltration method, micropillar porous silicon ciliated structure was used to separate the EVs. Our filtration membrane pore size was 0.01μm (100kDa MWCO Amicon® Ultra-2mL Centrifugal Filter,) which was capable of isolating the 40 to 100 nm EVs (Amedeo et al. 2014; Wang et al. 2013).

The method of Amedeo Cappione and Sara Gutierrez for centrifugal ultrafiltration was used with a slight modification. The cell culture medium was taken in the test tube and centrifuged to remove the cells and larger vesicles at 1000×g. After that, LNCaP cell culture medium was treated with androgen agonist R1881, antagonist MDV3100. The 2 mL of each from three samples were taken into a filtration tube and centrifuged 2000×g for 5 minutes in room temperature. The EVs were collected from the filtration membrane with PBS buffer.

3.9 Microscopy

3.9.1 Bright field microscopy

The bright field microscopy was used for observing the growth of the cell culture. An olympus GWB inverted light microscope (20X objectives) was used with phase-contrast illumination. Cell growth, confluency, any unwanted bacterial presence were checked every day with a bright field microscope.

3.9.2 Transmission electron microscopy

3.9.2.1 Fixation of EVs in electron grid with Negative staining

The fixation of EVs in an electron grid was prior to visualizing the EVs in an electron microscopy. We had followed the protocol of Théry et al., 2006, with a slight modification for negative staining of EVs in the electron grid. Firstly, the isolated EVs were resuspended with 50 μl of 2% PFA (Paraformaldehyde). The formvar-carbon coated EM grid (Electron

microscopy grid) was prepared by using a dehydrating vacuum pump for 20 seconds. Then 5µl of the sample given in the shiny side of the EM grid and waited for 20 minutes to absorb. PBS (Phosphate buffer saline) was placed in parafilm 50 µl each in three positions. The grid was transferred with clean forceps to wash three times in PBS. The shiny side of the grid was always kept wet where the sample was given and the opposite side was kept dry. The grid was transferred into 50 µl of 1% glutaraldehyde for 5 minutes. Then the EM grid was transferred for 2 minutes to a 50 µl drop of distilled water.

After 5 minutes, the EM grid was placed to a 5 µl drop of 0.05% UI (uranyl-oxalate) solution for 5 minutes. Then methyl cellulose-UA (uranyl acetate) 5 µl drop in Para-film kept over the ice was added with the gird for 10 minutes. Then air dries the grid for 5 to 10 minutes.

3.9.2.2 Immunogold labeling of whole-mount EVs

The immunogold labeling of EVs was another way of fixation for characterization of EVs in the electron microscopy. The fixation method was followed from Théry et al., 2006. The EVs were resuspended in 50µl of 2% PFA. The formvar-carbon grid was dehydrated for 20 seconds. Then 5µl of EVs sample were given in the grid and the grid was kept 20 minutes for drying. 50µl PBS was placed in three positions of parafilm and washed the grid. The grid was transferred into PBS/50 mM glycine for 3 minutes for three times. After that, the grid was transferred into blocking buffer, PBS/5 % (w/v) BSA for 10 minutes. The 5µl of antibody was added in the grid and was incubated for 30 minutes. The washing buffer (PBS/0.1 % (w/v) BSA) was used to wash for 3 minutes for five times. The grid was transferred into blocking buffer again for 3 min for five times.

The 5µl of protein A-gold conjugates was added to dilute in the blocking buffer for 20 minutes. The PBS was used to wash the EM grid and the gird was kept in 1% glutaraldehyde for 5 minutes. The 100µl water was used to rewash the EM grid for 2 minutes. After 5 minutes, the EM grid was relocated to a 5 µl drop of 0.05% UI (uranyl-oxalate) mixture. The 5µl methyl cellulose-UA (uranyl acetate) was added in parafilm kept over the ice for 10 minutes. The the grid was air dried for 5 to 10 minutes.

3.9.2.3 EVs observation in TEM

JEM-1400 Plus transmission electron microscopy was used for the EVs observation. The negatively stained EM grid was placed carefully for electron microscopy. The EM grid was observed in TEM at 80kv. The different shape of the EVs was revealed by the TEM.

The EVs were observed, focused and images taken with iTEM software. Images from iTEM software were saved as tif file format.

3.10 Image processing and statistical analysis

TEM images were processed with Image J software. Images of tif file format produced from iTEM software were imported into Image J software. Then the software was used to remove a bit of noise from the image.

Statistical data analysis was done with Microsoft Excel. The lectin coated Eu^{3+}-nanoparticles signal from Victor 2 were transferred in excel file. Then the average signal, the coefficient of variation, standard deviation, and signal by background was calculated from the counts. The two-dimensional graphs were represented by the difference between lectins binding fluorescence signals with EVs.

4. RESULTS

4.1 Standardization of TRF Immunoassay

At the beginning of our study, we had standardized the protocol with a serial dilution of the sample. Here in time-resolved fluorescence immunoassay, the biotinylated anti-CD9.C11 antibody was used as capture in 96 Streptavidin plate with assay buffer. *Ulex europaeus* agglutinin (UEA), Macrophage galactose-type lectin (MGL) and CD9-nanoparticles were used with five different dilutions of 12.5µl, 25 µl, 50 µl, 100 µl, 200 µl of LNCaP cell culture medium samples per well. TRF immunoassay standardization with five different amount dilutions of the sample and three nanoparticles tracer gives the graph of steady, gradual increase.

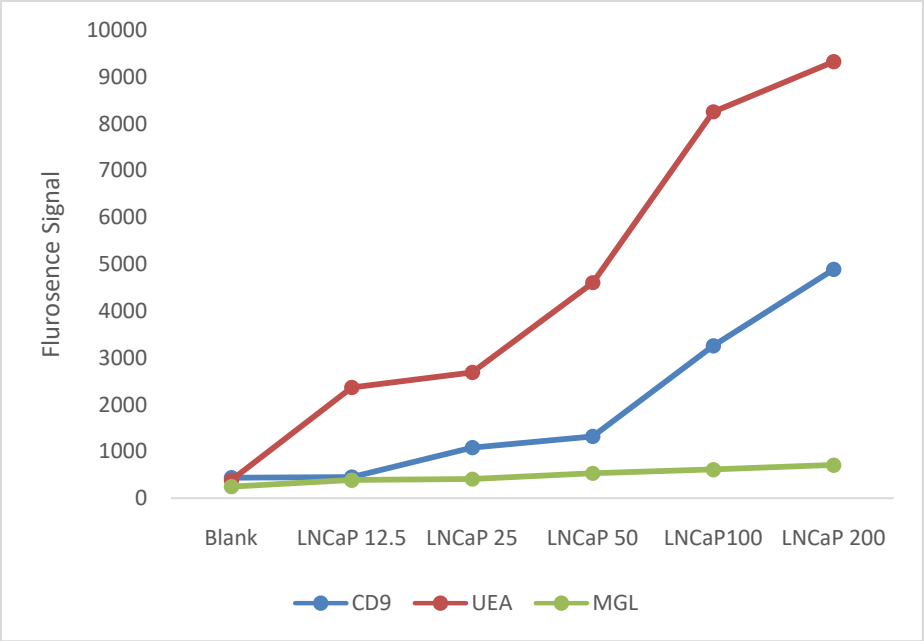


Figure 10: TRF Immunoassay protocol standardization with a serial dilution of LNCaP medium and different NPs tracers. Here the x-axis showed different samples amount of LNCaP cell culture medium sample and the y-axis showed the fluorescence signals.

4.2 PCa cell line selection

After standardization of the protocol, we have used four different PCa cell lines in combination with a panel of lectin-NPs tracer for our research. Among four types of cell line, LNCaP and VCaP cell lines were androgen sensitive and androgen insensitive cell lines were DU145 and PC3. The EVs isolated from these four cell lines were captured with biotinylated anti-CD9. Here ten lectins-NPs were used as the tracer to find out the best cell lines and lectin-NPs combination. We observed in figure 11 that UEA lectin gave a sharp rise of the signal in the LNCaP cell line.

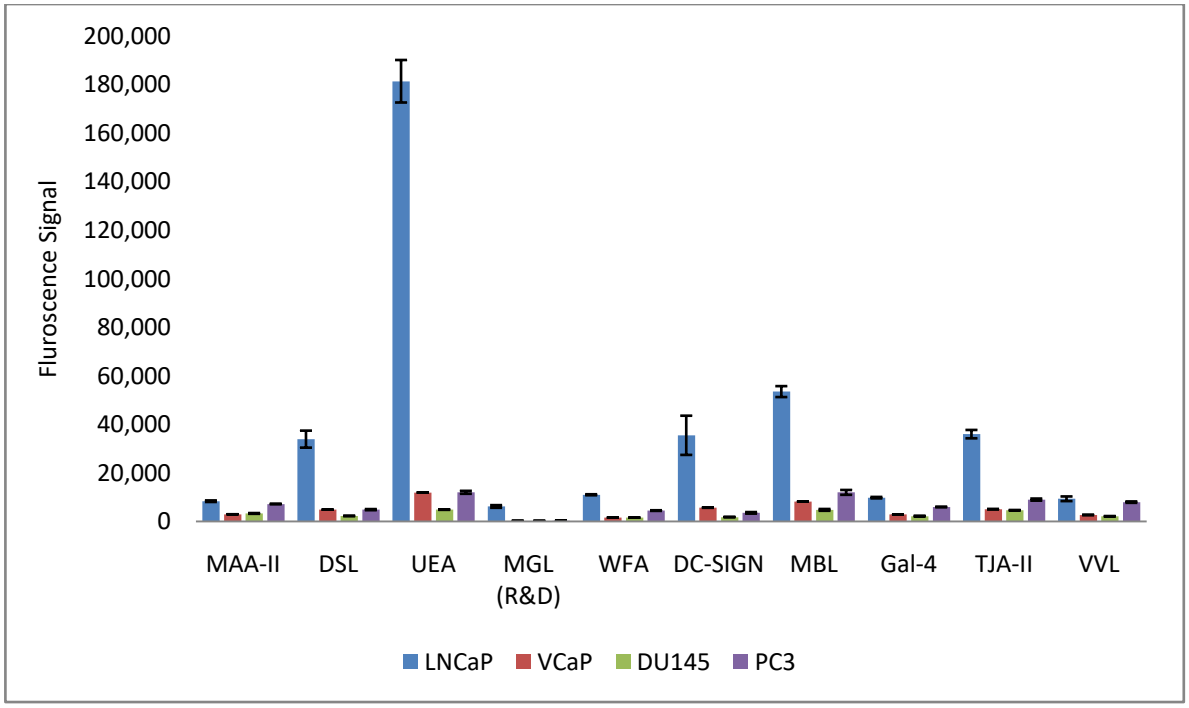


Figure 11: The PCa cell culture medium screening for the cell line selection from two androgen-sensitive (LNCaP, VCaP) and two androgen-insensitive cell line (DU145, PC3) with biotinylated CD9 capture antibody and ten different lectins-NPs. The x-axis showed the lectins and the y-axis showed the fluorescence signal.

4.3 Screening of lectins

The androgen positive LNCaP cell line showed higher signals compared to the other PCa cell lines in figure 11. Therefore, we had used LNCaP cell line for the rest of our experiment. LNCaP cells were treated with androgen receptor agonist R1881 and

antagonist MDV3100. Here biotinylated anti-CD9.C11 antibody was used as capture and 24 lectin-NPs were used to screen for binding with the EVs. Mainly plant source lectins used in our screening, but few animal source lectins and antibodies were also used here. In the figure 12, few lectin-NPs given sharp rise of fluroscence signals.

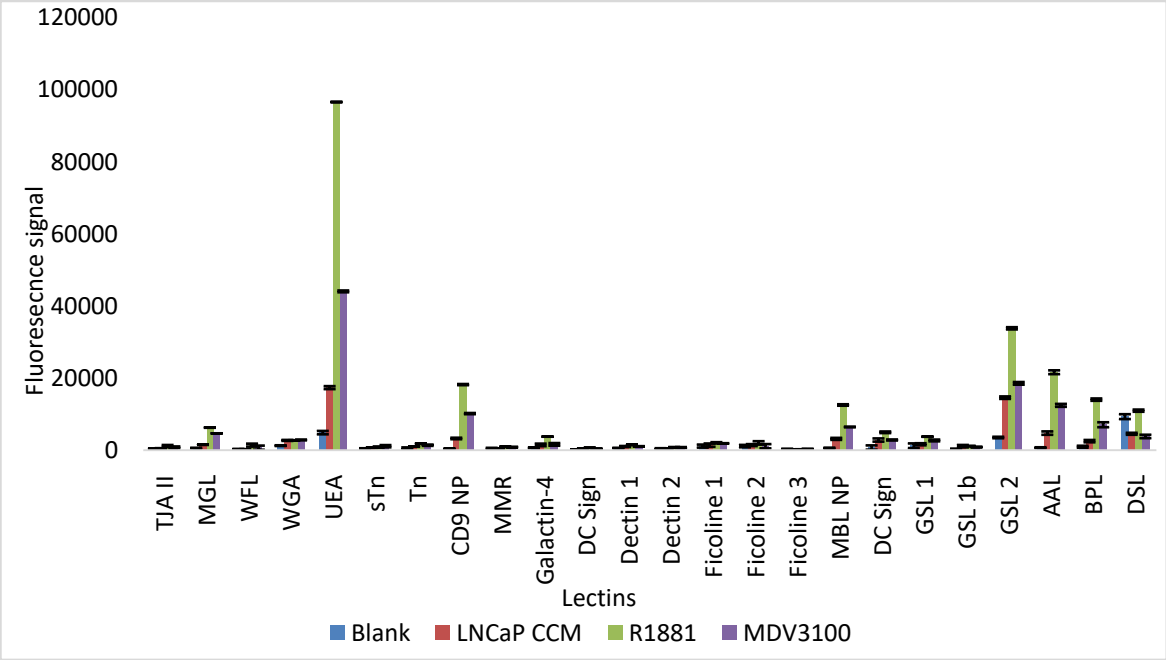


Figure 12: Screening of lectin-NPs with biotinylated CD9.C11 antibody and androgen receptor agonist R1881 and antagonist MDV3100 treated LNCaP cell culture medium. In this figure, the x-axis showed fluorescence signal and the y-axis presented lectins-NPs.

4.4 Different day production of treated LNCaP medium

Lectin-NPs approach was optimized with the different day collections of LNCaP cell culture medium which were treated with androgen receptor agonist R1881 and antagonist MDV3100. The LNCaP cell culture medium of day two, day five and day seven were collected. Then we had screened the samples with UEA lectin to identify best result for further EVs isolation. Biotinylated CD9.C11 antibody was used as capture. Here in the figure 13, day seven sample was given higher fluorescence signals.

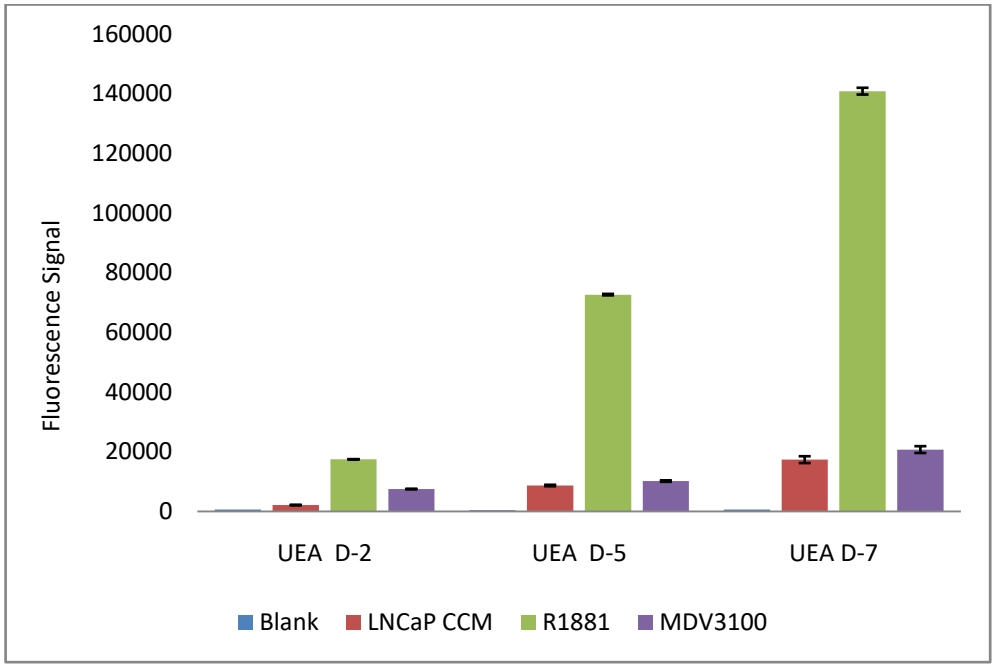


Figure 13: Screening of the samples from different day production with biotinylated CD9.C11 and UEA lectin. Here the x-axis represented the sample of day 2, 5, 7 respectively and the y-axis represented fluorescence signal.

4.5 Immunoassay with Isolated EVs

4.5.1 Isolated EVs immunoassay from ultracentrifugation method

Ultracentrifugation method is considered as the gold standard for the EVs isolation. Treated and untreated day seven production of the LNCaP cell culture medium was collected and the EVs were isolated. For the optimization of the sample amount, we used 200 ng, 400 ng, 800 ng, 1.6 µg, and 3.2 µg respectively. The CD9.C11 antibody was used and UEA lectin-NPs were used as tracer. In the figure 14, the 800 ng given higher fluroscence signal than other sample.

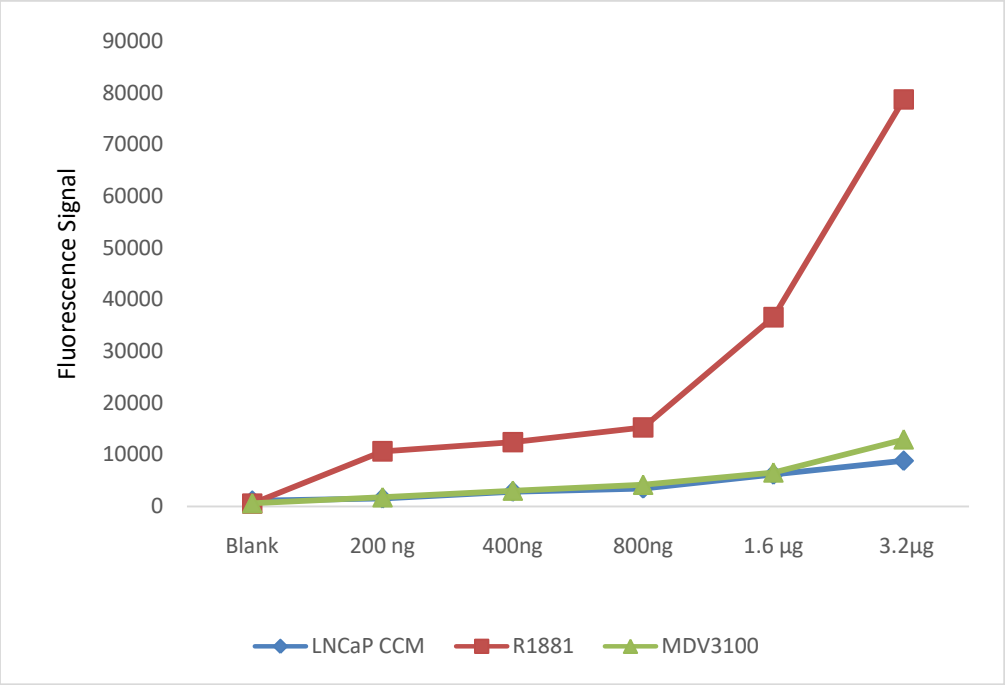


Figure 14: The isolated EVs sample amount variations were screened with biotinylated CD9.C11 and UEA lectin. The x-axis represented isolated EVs and the y-axis represented fluorescence signals.

We had chosen 800 ng isolated EVs for our lectin screening. We had found significant signal with UEA lectin. Here we also used CD9-NPs as the reference. Biotinylated anti-CD9.C11 was used as capture and UEA and CD9-NPs as the tracer for the experiment. Isolated EVs samples were already treated before isolation with ultracentrifugation. In the figure 15, UEA lectins showed higher signals with androgen receptor agonist R1881 treated EVs and androgen receptor antagonist MDV3100 treated EVs showed bit lower signals.

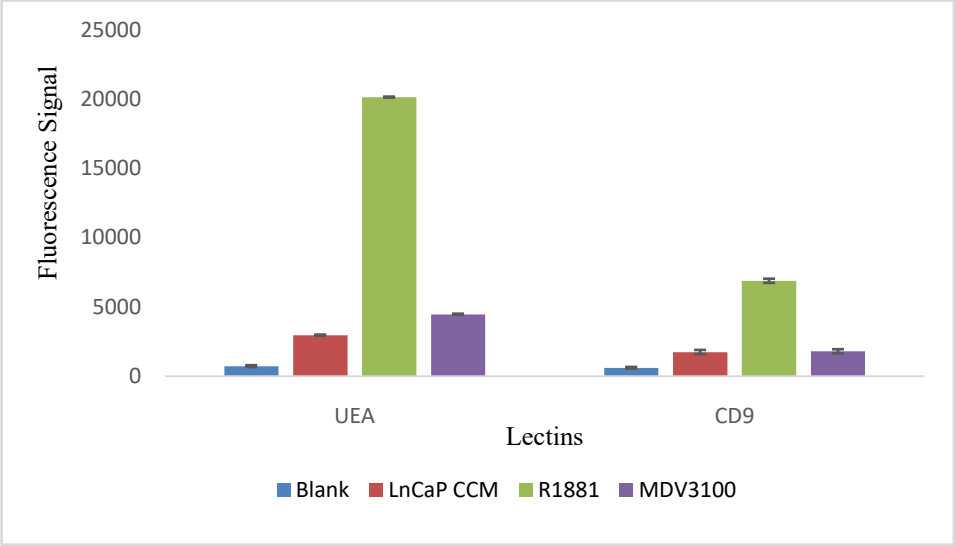


Figure 15: Screening of isolated EVs samples from the ultracentrifugation method with biotinylated CD9.C11 antibody and UEA, CD9 nanoparticles lectin. Here the x-axis represented lectins and the y-axis represented with signals.

4.5.2 Isolated EVs immunoassay from size exclusion chromatography

The size exclusion chromatography is another popular method of EVs isolation. But in our research, we did not find any significant signals from it. During immunoassay in SA plate, we had received very low fluroscence signals. Due to the technical difficulties with the concentration of cell culture medium, we did not manage to isolate enough EVs.

4.5.3 Isolated EVs immunoassay from ultrafiltration

Ultrafiltration is another method for the EVs isolation. We isolated the EVs from LNCaP cell culture medium treated with R1881, MDV3100 and untreated. The biotinylated CD9.C11 antibody was used as capture and two lectin-NPs used as the tracer. In figure 16, UEA lectin had significant higher signal with R1881 and MDV3100 treated sample showed one third lower signals than that.

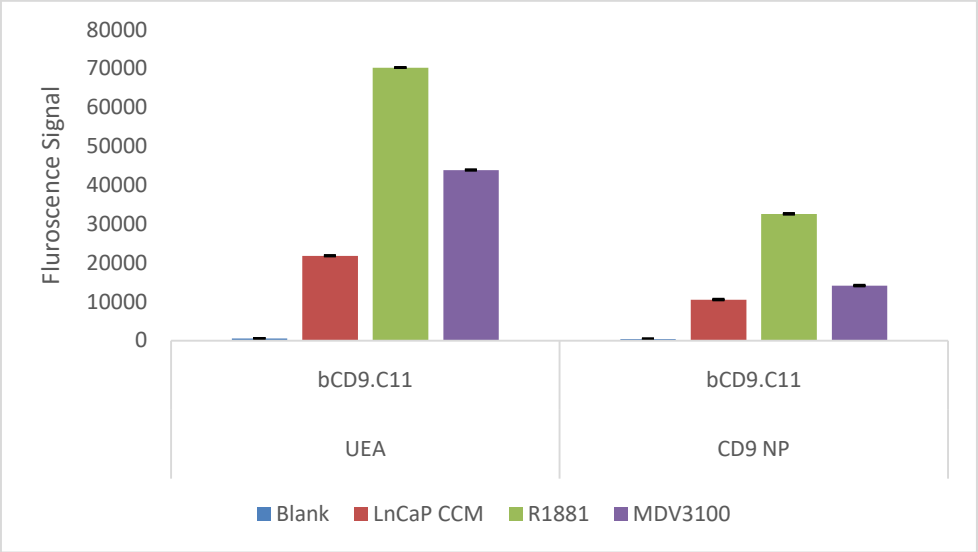
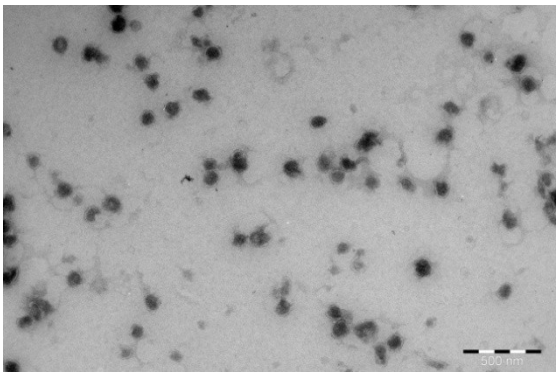


Figure 16: Screening of UEA and CD9-NPs lectins with isolated the EVs sample from ultrafiltration with biotinylated CD9.C11 antibody. Here the x-axis showed lectin and the y-axis represented signal from TRF-immunoassay.

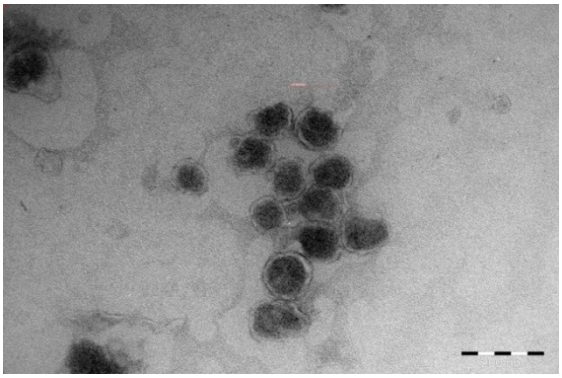
4.6 TEM Images of EVs

The EVs from LNCaP and VCaP cell line images were taken from TEM. In electron microscopy, we saw the double layer oval or circular shape of EVs about 100 nm. Isolated pure EVs from LNCaP and VCaP were diluted and negatively stained for TEM.

The diameter of most EVs is near about 100nm in TEM images. The scale bar was added in every image from iTEM software while images were taken. From figure 17 to figure 20 were from isolated EVs of LNCaP cells and figure 21 to figure 23 were taken from VCaP cell line. Although we tried to take the image from immune gold stained LNCaP cell line EVs but failed to see any of the EVs. There are some smaller cellular fractions was visible which was ranging 10nm to 30nm.

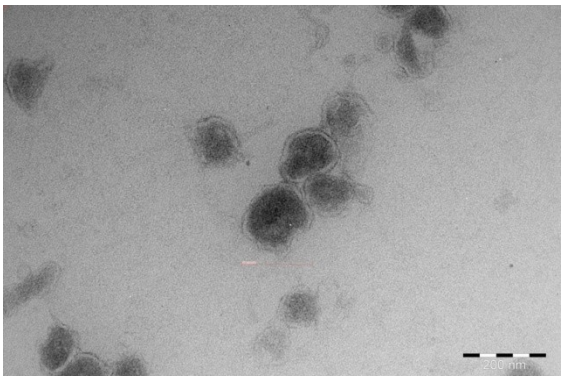


(Figure 17)

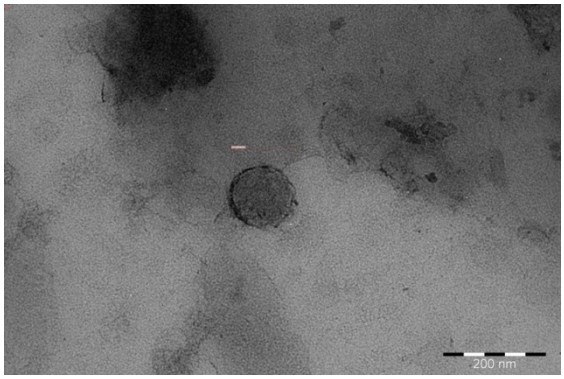


(Figure 18)

Figure 17 & 18: Isolated EVs of LNCaP cell line from negative fixation in TEM. In figure 17, the scale of 500nm was used and small double layer EVs were easily visible. In figure 18, a cluster of double layer EVs of LNCaP cells visible in the scale of 200nm.

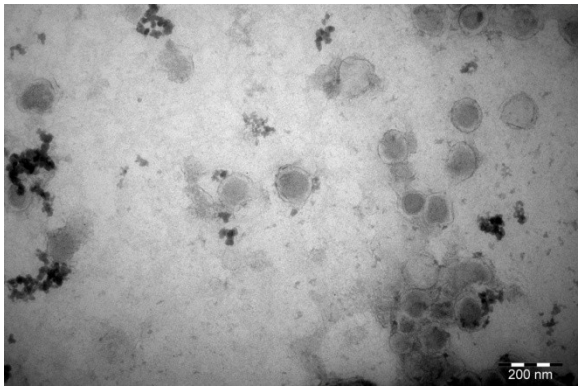


(Figure 19)

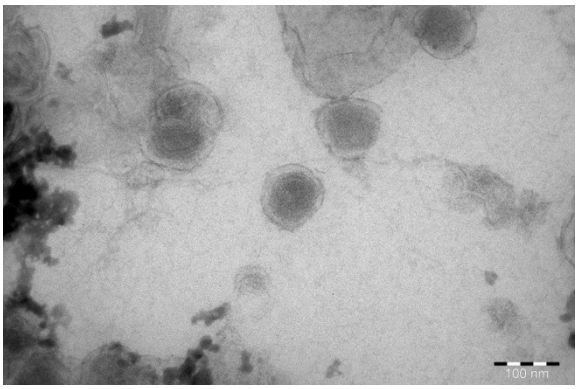


(Figure 20)

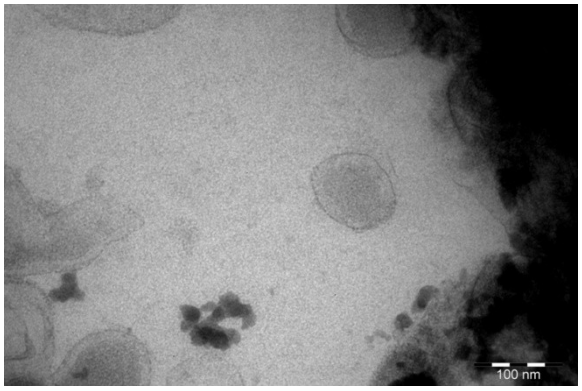
Figure 19&20: Isolated EVs of LNCaP cell line from the negative fixation in TEM. In figure 19, double layer and round shaped EVs were visible in scale of 200nm. In figure 20, here single EV visible which had length of 127.77nm under the scale of 200nm.



(Figure 21)



(Figure 22)



(Figure23)

Figure 21, 22 & 23: Isolated EVs from VCaP cell line with negative fixation in TEM. In figure 21, in the scale of 200nm VCaP EVs visible with double layer. In the figure 22 & 23, the scale of 100 nm VCaP EVs were visible.

5. DISCUSSION

Glycosylation and androgen hormone activity has a direct link with PCa. The clinically cellular level glycosylation enzymes and their relation with androgen hormone play an essential role in the progression of prostate cancer (Munkley, 2017). In our study, we introduced lectin nanoparticle approaches for the identification of glycosylation of EVs from the androgen-treated and untreated LNCaP cell line. In LNCaP-EVs, we observed that the signal was increased by androgen hormone agonist (R1881) and reduced by androgen hormone antagonist (MDV3100).With our lectin library screening, we found a fucose-binding lectin UEA, which showed that the signal for binding fucosylation was increased by R1881 treated EVs and reduced by MDV3100.

Lectin nanoparticle immunoassay enhances the binding affinity of the lectin to glycan through the avidity effect with signal amplification (Gidwani et al., 2016; Kekki et al., 2016). In our research, we used biotinylated anti-CD9, anti-CD9.C11 antibodies as capture EVs and nanoparticle-coated lectin to detect altered glycosylation on the surface of EVs. We have used androgen-hormone positive PCa cell lines (LNCaP, VCaP) and androgen-hormone negative PCa cell lines (DU145, PC3).Among these four cell lines. Androgen positive LNCaP cell line gives a higher signal than other cell lines. Thus, we have chosen the LNCaP cell line, subsequently cultured and treated with androgen receptor agonist R1881 and androgen receptor antagonist MDV3100. Twenty-four Eu^{3+}- coated lectin nano-particles were screened where UEA lectin NPs gives a higher signal than any other lectins. TEM images of EVs clarify the double lipid layer and size 50 to 200nm. However, glycosylation changes on the surface of EVs cannot be seen in TEM from immune-gold fixation. EVs were collected and characterized by different methods. In ultracentrifugation and ultrafiltration methods, UEA lectin was given similar results and CD9 nanoparticle used as the reference.

It was known that the secretion and expression of CD9 in EVs increased by the treatment with AR (androgen receptor) agonist R1881 (Chuan et al., 2005). As the same amount of isolated EVs given in each well and according to the Chuan's paper CD9 in EVs secretion and expression increased by AR agonist; so UEA lectin binds with the glycan moieties of EVs and given higher signal than other samples (Chuan et al., 2005).

UEA lectin usually binds with fucose linked α1, 2 to Gal (Sato et al., 1986; Orczyk-Pawilowicz et al., 2013). Fucosylation of glycoproteins recognized fucosylated oligosaccharides which were used as tumor markers. Growth factor receptors and adhesion molecules are regulated by fucosylation of glycoprotein (Miyoshi et al., 2008). Thus, fucosylation of EVs glycan by UEA lectin indicates prostate cancer presences. This clinical evidence can further lead to inventing new biomarkers for prostate cancer.

Soekmadji et al., 2016 showed that PCa progression is contributed by CD9-positive EVs which are involved in mediating paracrine signaling. It was also confirmed from the assay in the paper that AR agonist treated LNCaP cells showed significant development in the production of CD9 positive EVs. AR agonist-treated cells EVs which are enriched with CD9 can influence the growth in the androgen-deprived situation (Soekmadji et al., 2016). In our study, we found that the fluorescence signal was higher with EVs from DHT-treated LNCaP cells which indicated similar results.

Altered expression of glycosylation enzyme triggered altered glycosylation; which resulted in glycan modification, cell-cell adhesion, migration, signaling and influence PCa progression (Munkley et al., 2016). Androgen regulates the process of glycosylation because during androgen-deprived therapy (ADT) glycosylation enzymes lost their activity (Munkley et al., 2016). So in our study, we find a similar activity for EVs altered glycosylation. AR agonist (R1881) treated EVs gives a significant difference in its fluorescence signal than AR antagonist (MDV3100) treated EVs.

It is already known that glycosylation changes of EVs are a common phenomenon in cancer and altered glycans in EVs are the potential diagnostic target for cancer (Satomaa et al., 2009, Gerlach et al., 2017). Thus fucosylation of UEA lectin gives hints for future diagnostic tools for prostate cancers.

This lectin nanoparticle-immunoassay can play an essential role for inventing more specific and accurate diagnostic approach than current diagnostic methods. Androgen-regulated glycosylation of EVs identification by UEA lectin possibly be applied in further research for inventing non-invasive and more specific diagnostic method. From this simple affinity assay, our finding discloses a correlation between androgen hormone regulation and glycosylation of surface EVs.

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